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Inhibitory actions of diphenyleneiodonium on endothelium-dependent vasodilatations *in vitro* and *in vivo*

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1 This study examined the *in vitro* and *in vivo* inhibitory effects of diphenyleneiodonium (DPI), a novel inhibitor of nitric oxide (NO) synthase, on endothelium-dependent vasodilatations.

2 DPI (3×10^{-8}–3×10^{-6} M) concentration-dependently inhibited acetylcholine (ACh)-induced relaxation in preconstricted rat thoracic aortic rings, with an IC_{50} of 1.8×10^{-7} M and a maximal inhibition of nearly 100%. DPI (3×10^{-6} M) also completely inhibited the relaxation induced by the calcium ionophore, A23187 but not by sodium nitroprusside (SNP). The inhibitory effect of DPI (3×10^{-7} M) on ACh-induced relaxation was prevented by pretreatment with NADPH (5×10^{-3} M) and FAD (5×10^{-4} M) but not L-arginine (L-Arg, 2×10^{-3} M). Pretreatment with NADPH did not alter the inhibitory effect of N^G-nitro-L-arginine on ACh-induced relaxation.

3 The inhibitory effect of DPI on ACh-induced relaxation in the aortae lasted >4 h after washout. In contrast to pretreatment, post-treatment (1 h later) with NADPH (5×10^{-3} M) reversed only slightly the inhibitory effect of DPI.

4 In conscious rats, DPI (10^{-5} mol kg^{-1}) inhibited the depressor response to i.v. infused ACh, but not SNP. However, it caused only a transient pressor response which was previously shown to be due completely to sympathetic activation.

5 Thus, DPI is an efficacious and 'irreversible' inhibitor of endothelium-dependent vasodilatation *in vivo* and *in vitro*. The mechanism of the inhibition may involve antagonism of the effects of FAD and NADPH, co-factors of NO synthase. However, unlike the N^G-substituted arginine analogues (another class of NO synthase inhibitors), DPI-induced suppression of endothelium-dependent vasodilatation *in vivo* does not lead to a sustained rise in blood pressure.

Keywords: Diphenyleneiodonium (DPI); nitric oxide synthase inhibitor; endothelium-dependent relaxation; blood pressure; acetylcholine (ACh); A23187; sodium nitroprusside (SNP); FAD

Introduction

A group of iodonium compounds have been reported to be a new class of nitric oxide (NO) synthase inhibitors in the macrophage (Stuehr *et al.*, 1991b; Kwon *et al.*, 1991; Keller *et al.*, 1992). These compounds include diphenyleneiodonium (DPI), iodoniumdiphenyl and di-2-thienyliodonium, all of which have chemical structures distinct from those of N^G-substituted arginine (Arg) analogues. DPI was initially found to be a potent hypoglycaemic agent (Stewart & Hanley, 1969; Gatley & Martin, 1979) which, by inhibiting gluconeogenesis from lactate and aspartate, suppressed the oxidation of NADH-linked substances (Holland *et al.*, 1973). It was later shown that DPI, iodoniumdiphenyl and di-2-thienyliodonium suppressed the activities of neutrophil and macrophage NADPH-dependent oxidase (Cross & Jones, 1986; Hancock & Jones, 1987; Ellis *et al.*, 1988; 1989), probably *via* inhibition of a flavoprotein (Cross & Jones, 1986; Hancock & Jones, 1987; Ellis *et al.*, 1989; O'Donnell *et al.*, 1993).

The pharmacology of N^G-substituted Arg analogues, which include N^G-monomethyl-L-Arg (L-NMMA), N^G-nitro-L-Arg (L-NOARG), N^G-nitro-L-Arg methyl ester (L-NAME), L-iminoethyl-ornithine (L-NIO) and N^G-amino-L-Arg (L-NAA) (see Moncada *et al.*, 1991), has been extensively studied. These compounds cause sustained inhibition of endothelium-dependent relaxation *in vitro* and produce prolonged pressor responses in whole animals (Aisaka *et al.*, 1989; Rees *et al.*, 1989; 1990; Wang & Pang, 1990; Wang *et al.*, 1991; 1992; 1993; Pang & Wang, 1993). The pressor effects of these compounds have been attributed to the inhibition of the L-Arg/NO pathway and endothelium-dependent vasodilatation *in situ* (Aisaka *et al.*, 1989; Rees *et al.*, 1989; see Moncada *et al.*, 1991). Accordingly, DPI being an inhibitor of NO synthase, would be expected to cause a pressor response in whole animals. Indeed, i.v. injections of DPI produced transient pressor responses in pentobarbitone-anaesthetized and conscious rats (Wang & Pang, 1993a,b). However, unlike that of the N^G-substituted Arg analogues (Wang & Pang, 1991), the pressor effect of DPI was attenuated or abolished by blockers of the sympathetic nervous system (Wang & Pang, 1993a,b).

The reasons for the differences in the causative factor and time course of the pressor responses elicited by these two classes of NO synthase inhibitors are unclear. One possibility is that DPI does not produce prolonged inhibition of endothelium-dependent vasodilatations *in vitro* and/or *in vivo*. Another possibility is that inhibition of endothelium-dependent vasodilatation by DPI is not the cause of the elevation of blood pressure. Hence, the first aim of this study was to find out if DPI causes prolonged *in vitro* and *in vivo* inhibition of endothelium-dependent vasodilatations, as DPI has been shown to cause prolonged inhibition of NO biosynthesis in macrophages (Stuehr *et al.*, 1991b). This was assessed by studying the effects of DPI on relaxations induced by the endothelium-dependent vasodilators, acetylcholine (ACh) and A23187 (calcium ionophore), and the endothelium-independent vasodilator sodium nitroprusside (SNP) in preconstricted rat aorta. In addition, the effects of DPI on depressor responses to ACh and SNP were studied in conscious, unrestrained rats. The second aim was to determine if slower onset pressor responses (other than the initial transient rise in blood pressure) followed the administration of DPI. The third aim was to examine if the NO synthase co-factors NADPH and FAD, as well as the NO synthase substrate L-Arg, reversed the inhibitory effect of DPI on endothelium-dependent relaxation.

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Methods

Male Sprague-Dawley rats (350–420 g) were used in this study.

Isolated aortic rings

The rats were killed by a blow on the head followed by exsanguination. The thoracic aorta was removed and cleared of connective tissue. Four ring segments of 0.5 cm length were prepared from one aorta and suspended in random order in separate organ baths. Each ring was connected to a Grass FT-03-C force-displacement transducer for isometric recording with a preload of 1 g. The rings were equilibrated for 1 h (with 3 washouts) in Krebs solution (pH 7.4) at 37°C and bubbled with a gas mixture of 95% O_2 and 5% CO_2. The Krebs solution had the following composition (mM): NaCl 118, glucose 11, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgCl_26H_2O$ 1.2.

The rings were first incubated with vehicle or drugs (see later) followed by phenylephrine (PE, 10^{-6} M, EC_{90}). After 15–20 min, at the steady-state phase of the contractile response to PE, a cumulative concentration-response curve to ACh, A23187 (calcium ionophore) or SNP was obtained. Each drug concentration was left in the bath until a plateau response was reached. The time taken to complete each concentration-response curve was approximately 20 min. In groups where more than one concentration-response curve of ACh was constructed, the preparations were washed three times within 30 min and given another 30 min to recover completely from the effects of the previous applications of PE and ACh. Afterwards, PE was again added followed by the construction of ACh concentration-response curves.

Conscious rats

The rats were anaesthetized with halothane (4% in air for induction and 1.2% in air for surgery). Polyethylene cannulae (PE50) were inserted into the left iliac artery for the measurement of mean arterial pressure (MAP) by a pressure transducer (P23DB, Gould Statham, CA, U.S.A.) and into the left iliac vein for the administration of drugs. The cannulae were filled with heparinized (25 iu ml^{-1}) normal saline, tunnelled s.c. along the back and exteriorized at the back of the neck. The rats were put into small cages allowing free movement and given >6 h recovery from the effects of surgery and halothane before use.

Drugs

The following drugs were purchased from Sigma Chemical Co. (MO, U.S.A.): acetylcholine (ACh) chloride, A23187, phenylphrine (PE) hydrochloride, flavin adenine dinucleotide (FAD) disodium, β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), N^{ω}-nitro-L-arginine (L-NOARG) and L-arginine (L-Arg) hydrochloride. Diphenyleneiodonium (DPI) sulphate and sodium nitroprusside (SNP) were obtained from Colour Your Enzyme Ltd. (Ontario, Canada) and Fisher Scientific Co. (N.J., U.S.A.), respectively. All drugs were dissolved in normal saline (0.9% NaCl) except for DPI and A23187 which were dissolved in 5% glucose solution and 10% dimethyl sulphoxide, respectively.

Experimental protocols

Each experiment included 6–8 aortic rings or 6 conscious, unrestrained rats.

Protocol 1: Effects of DPI on ACh-, A23187- and SNP-induced relaxations in the aorta Six groups of aortic rings were incubated with the vehicle or DPI (3×10^{-8}–3×10^{-6} M) followed by PE 10 min later. After another 10–15 min, concentration-response curves to ACh (10^{-8}–3×10^{-5} M) were obtained. Only one concentration of DPI was studied in each group. Another two groups were treated with the vehicle or DPI (3×10^{-6} M) followed by the application of PE and construction of concentration-response curve to A23187 (10^{-9}–10^{-6} M). The last two groups were treated in the same way as the previous two groups except that SNP (3×10^{-10}–10^{-7} M) was used in place of A23187.

Protocol 2: Effects of pretreatment with NADPH, FAD or L-Arg on the inhibitory effect of DPI Twelve groups of aortic rings were treated with vehicle + vehicle, NADPH (1.5×10^{-3} M) + vehicle, NADPH (5×10^{-3} M) + vehicle, FAD (5×10^{-6} M) + vehicle, FAD (5×10^{-4} M) + vehicle and L-Arg (2×10^{-3} M) + vehicle, vehicle + DPI, NADPH (1.5×10^{-3} M) + DPI, NADPH (5×10^{-3} M) + DPI, FAD (5×10^{-6} M) + DPI, FAD (5×10^{-4} M) + DPI, and L-Arg (2×10^{-3} M) + DPI, with 3×10^{-7} M DPI added in all cases. Another two groups of aortic rings were treated with vehicle + L-NOARG (10^{-6} M) and NADPH (5×10^{-3} M) + L-NOARG (10^{-6} M). The first treatments were given 10 min prior to the second treatments. Afterward, the rings were preconstricted with PE and relaxed with ACh as described in Protocol 1.

Protocol 3: Time course and reversibility of the inhibitory effect of DPI on relaxation response of ACh The time course of the inhibitory effect of DPI was studied in 3 groups of aortic rings. After completing the first concentration-response curve to ACh in PE-preconstricted rings in the presence of vehicle or DPI (3×10^{-7} or 3×10^{-6} M), the preparations were washed out without further addition of drug or vehicle. Second, third and fourth ACh curves were constructed in preconstricted rings at 1.5, 4 and 9 h after the preparations were washed out. In another two groups of aortic rings, the effect of post-treatment with NADPH on the inhibitory effect of DPI was studied. These rings were incubated with DPI (3×10^{-7} M) or vehicle for 1 h, followed by the application of NADPH (5×10^{-3} M) and the construction of ACh concentration-response curves in PE-preconstricted conditions.

Protocol 4: Effect of DPI on resting MAP and depressor response to ACh and SNP In one group of rats, DPI (10^{-5} mol kg^{-1}) was injected (i.v. bolus) and blood pressure was continuously monitored for 2 h.

Two groups of rats were injected (i.v. bolus) with the vehicle or DPI (10^{-5} mol kg^{-1}) 20 min prior to i.v. infusions of ACh (6×10^{-8}–1.8×10^{-6} mol kg^{-1} min^{-1}, each dose for 4 min) and SNP (3×10^{-8}–4.8×10^{-7} mol kg^{-1} min^{-1}, each dose for 4 min). The sequence of ACh and SNP administrations was reversed in half of the studies with 20 min recovery after the completion of the first dose-response curve. The time taken to complete the experiment was approximately 2 h after i.v. injection of DPI or the vehicle.

Calculation and statistical analysis

IC_{50} and E_{max} were calculated from individual concentration-response curves (see Wang & Pang, 1993a). All results are expressed as mean ± standard error (s.e.mean) except for the points where the error bars were smaller than the symbols (see figures). The results were analysed by the analysis of variance/co-variance followed by Duncan's multiple range test with $P<0.05$ selected as the criterion for statistical significance.

Results

Effects of DPI on ACh, A23187- and SNP-induced relaxations

All five concentrations of DPI (3×10^{-8}, 10^{-7}, 3×10^{-7}, 10^{-6} and 3×10^{-6} M) slightly potentiated PE-induced contraction

from the baseline value of 0.99 ± 0.10 g to 1.09 ± 0.15, 1.27 ± 0.11, 1.32 ± 0.10, 1.31 ± 0.10 and 1.18 ± 0.14 g, respectively. However, only the effects of the third and fourth concentrations of DPI were statistically significant.

In the vehicle-treated group, ACh relaxed the preconstricted aorta concentration-dependently with maximum relaxation of approximately 60% (Figure 1a). DPI inhibited concentration-dependently the ACh-induced relaxation. At 3×10^{-5} M ACh, the IC_{50} of DPI was 1.8×10^{-7} M with a maximum inhibition of 96% (Figure 1b).

In another two vehicle groups, A23187 and SNP also relaxed concentration-dependently the preconstricted aortae, with maximal relaxations of approximately 60 and 100%, respectively. DPI (3×10^{-6} M) completely inhibited A23187-induced relaxation (Figure 2a) but did not affect the relaxation response of SNP (Figure 2b).

Influences of NADPH, FAD and L-Arg on the inhibitory effects of DPI and, the effect of L-NOARG on ACh-induced relaxation

Baseline contractions elicited by PE in the presence of vehicle or DPI (3×10^{-7} M) were 1.29 ± 0.07 and 1.67 ± 0.12 g, respectively. Treatment with NADPH (1.5 and 5×10^{-3} M), FAD (5×10^{-4} and 5×10^{-6} M) and L-Arg (2×10^{-3} M) did not significantly affect PE-induced contractions in the presence of either the vehicle (1.04 ± 0.06, 1.04 ± 0.11, 1.16 ± 0.06, 1.33 ± 0.11, 1.23 ± 0.08 g, respectively) or DPI (1.43 ± 0.10, 1.37 ± 0.14, 1.59 ± 0.06, 1.52 ± 0.13, 1.44 ± 0.04 g, respectively).

DPI inhibited ACh-induced relaxations (Figure 3a). Treatment with L-Arg did not affect either the ACh-induced

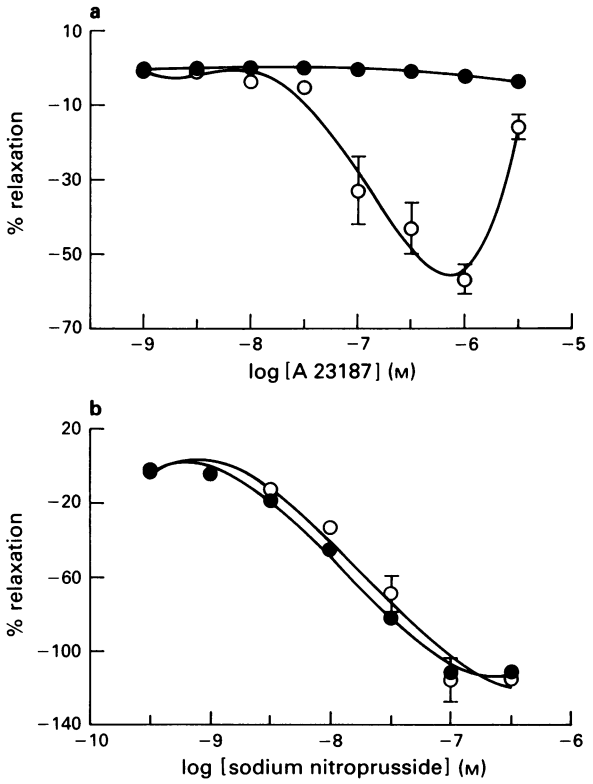

Figure 2 Effects (mean ± s.e.mean) of vehicle (○) or diphenyleneiodonium (DPI, 3×10^{-6} M, ●) on A23187 (a) and sodium nitroprusside (b) induced relaxations in the phenylephrine (10^{-6} M) preconstricted aortic rings ($n = 6$ each group). *Significant difference from vehicle-pretreated control curve ($P < 0.05$).

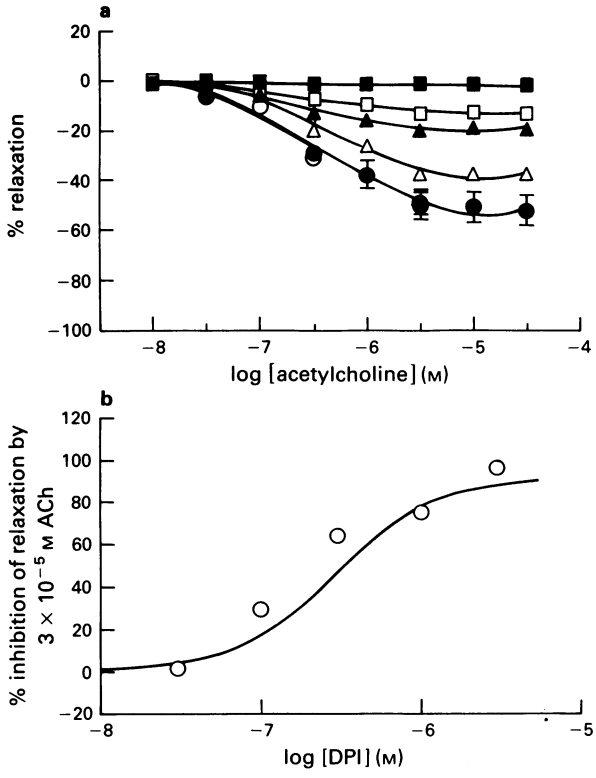

Figure 1 (a) Concentration-response (mean ± s.e.mean) curves of diphenyleneiodonium (DPI) on acetylcholine-induced relaxation in phenylephrine (10^{-6} M)-preconstricted aortic rings ($n = 7$ each group). Concentrations of DPI were as follows: vehicle (○); 3×10^{-8} M (●); 10^{-7} M (△); 3×10^{-7} M (▲); 10^{-6} M (□); 3×10^{-6} M (■). (b) Percentage inhibition by DPI of 3×10^{-5} M acetylcholine-induced relaxation in the aorta. The data were derived from the mean values of (a).

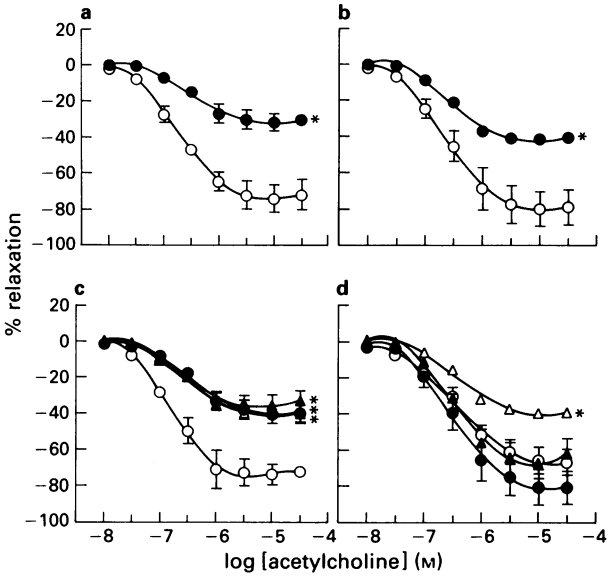

Figure 3 Effects (mean ± s.e.mean) of vehicle, NADPH, FAD and L-arginine on acetylcholine-induced relaxation and on the inhibitory influence of diphenyleneiodonium (DPI, 3×10^{-7} M) on relaxation in phenylephrine (10^{-6} M), preconstricted aortic rings ($n = 6$ each group). The first treatments were performed 10 min before the second treatments. (a) Vehicle + vehicle (○); vehicle + DPI (●). (b) L-Arginine (2×10^{-3} M) + vehicle (○); L-arginine (2×10^{-3} M) + DPI (●). (c) FAD (5×10^{-6} M) + vehicle (○); FAD (5×10^{-4} M) + vehicle (●); FAD (5×10^{-6} M) + DPI (△); FAD (5×10^{-4} M) + DPI (▲). (d) NADPH (1.5×10^{-3} M) + vehicle (○); NADPH (5×10^{-3} M) + vehicle (●); NADPH (1.5×10^{-3} M) + DPI (△); NADPH (5×10^{-3} M) + DPI (▲). *Significant difference from control curve ($P < 0.05$).

relaxation or the inhibitory effect of DPI on ACh-induced relaxation (Figure 3b). Although the lower concentration (5×10^{-6} M) of FAD also did not alter either ACh-induced relaxation or the inhibitory effect of DPI on ACh, the higher concentration (5×10^{-4} M) of FAD suppressed the relaxant effect of ACh and prevented further inhibition by DPI on ACh-induced relaxation (Figure 3c). Although neither concentration of NADPH significantly affected ACh-induced relaxation, the higher (5×10^{-3} M) but not the lower (1.5×10^{-3} M) concentration completely prevented the inhibitory effect of DPI (Figure 3d). The effectiveness of pretreatment with NADPH (5×10^{-3} M) in inhibiting the effect of DPI, expressed as the ratio of the relaxation effect of 10^{-5} M ACh in the presence of NADPH (−67%) to that in the absence of NADPH (−32%), was 209%.

PE caused contractions of 1.46 ± 0.12 and 1.67 ± 0.13 g in the presence of vehicle + L-NOARG (10^{-6} M) and NADPH (5×10^{-3} M) + L-NOARG (10^{-6} M), respectively. Compared to the pooled vehicle control derived from Figures 1a and 3a, L-NOARG markedly inhibited ACh-induced relaxation. Pretreatment with NADPH did not affect the inhibitory effect of L-NOARG (Figure 4).

Time course and reversiblity of the inhibitory effect of DPI on ACh-induced relaxation

The PE-induced contractions in the presence of vehicle or DPI did not change with the passage of time (data not shown). The ACh-induced maximal relaxation was not altered until at least 4 h after washout. Maximal relaxation at 9 h was $-48 \pm 7\%$, which was significantly less than that ($-69 \pm 6\%$) at 0 h (Figure 5). DPI at 3×10^{-7} and 3×10^{-6} M inhibited ACh-induced relaxation by approximately 50 and 100%, respectively (Figure 5a). The inhibitory effect of DPI remained at least 4 h after washout (Figure 5b,c). At 9 h after washout, the relaxations of DPI-pretreated rings were still less, though insignificantly, than those of vehicle-pretreated rings (Figure 5d).

Maximum relaxation to ACh after 1 h exposure to 3×10^{-7} M DPI ($-38.3 \pm 3.1\%$, Figure 6) was similar to that after a 10 min exposure to DPI ($-32.2 \pm 4.8\%$, Figure 3a). Post-treatment (1 h later) with NADPH (5×10^{-3} M) slightly but significantly, suppressed the inhibitory effect of DPI. The effectiveness of post-treatment with NADPH (5×10^{-3} M) in inhibiting the effect of DPI, expressed as a ratio of the relaxation effect of 10^{-5} M ACh in the presence of NADPH (−51%) to that in the absence of NADPH (−38%), was 134% (Figure 6).

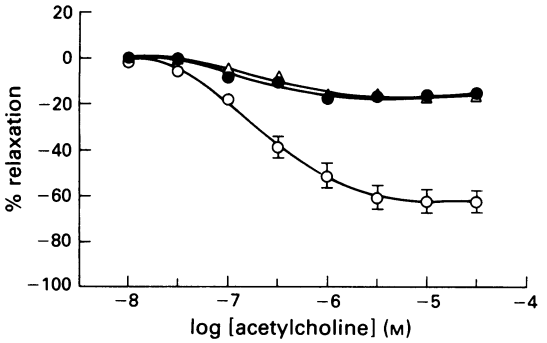

Figure 4 Effect (mean ± s.e.mean) of pretreatments (10 min earlier) of NADPH (5×10^{-3} M) on the inhibitory effect of N^G-nitro-L-arginine (L-NOARG, 10^{-6} M) on acetylcholine-induced relaxation in the phenylephrine (10^{-6} M) preconstricted aortic rings ($n = 8$ each group except for the pooled control rings where $n = 13$). Vehicle (○); vehicle + L-NOARG (●); NADPH + L-NOARG (△). *Significant difference from vehicle-pretreated control curve ($P < 0.05$).

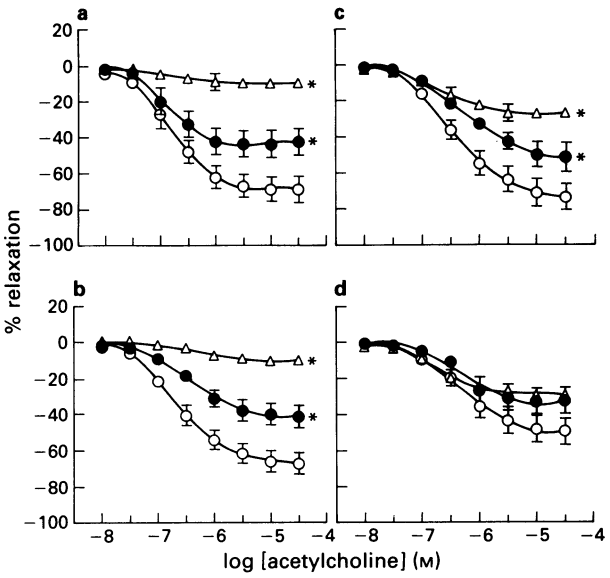

Figure 5 The time course of the effects (mean ± s.e.mean) of vehicle (○), diphenyleneiodonium (DPI, 3×10^{-7} M, ●) and DPI (3×10^{-6} M, △) on acetylcholine-induced relaxations in phenylephrine (10^{-6} M) preconstricted aortic rings ($n = 6$ each group). (a), (b), (c) and (d) represent responses at 0, 1.5, 4 and 9 h after washout without further addition of the vehicle or DPI. *Significant difference from vehicle-pretreated control curve ($P < 0.05$).

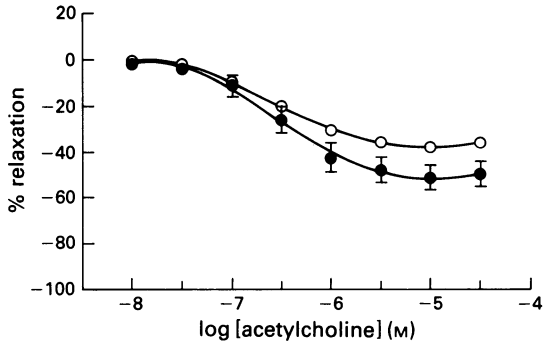

Figure 6 Effects (mean ± s.e.mean) of post-treatment (1 h later) with vehicle (○) or NADPH (5×10^{-3} M, ●) on the inhibitory effect of diphenyleneiodonium (DPI, 3×10^{-7} M) on acetylcholine-induced relaxation in the phenylephrine (10^{-6} M) preconstricted aortic rings ($n = 6$ each group). *Significant difference from vehicle-pretreated control curve ($P < 0.05$).

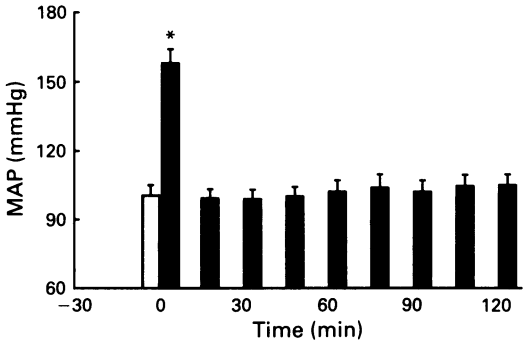

Figure 7 Effect (mean ± s.e.mean) of i.v. bolus injections of diphenyleneiodonium (DPI, 10^{-5} mol kg^{-1}) on mean arterial pressure (MAP) in conscious rats ($n = 6$). Open and solid columns represent pre- and post-administration with DPI. *Significant difference from pre-administration with DPI ($P < 0.05$).

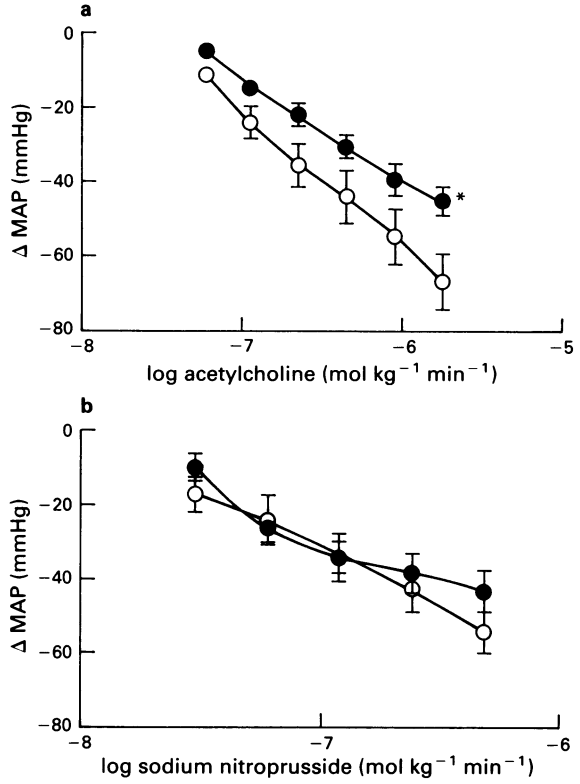

Figure 8 Dose-response curves (mean ± s.e.mean) of i.v. infusions of acetylcholine (a) and sodium nitroprusside (b) on mean arterial pressure (MAP) in conscious rats ($n = 6$ each group) pretreated with i.v. bolus injection of vehicle (○) or diphenyleneiodonium (DPI, 10^{-5} mol kg^{-1}, ●). *Significant difference from vehicle-pretreated control curve ($P < 0.05$).

Effects of DPI on resting blood pressure and depressor responses to ACh and SNP

Intravenous bolus injections of DPI (10^{-5} mol kg^{-1}) in conscious rats caused immediate and transient increases in MAP which were similar to the responses in pentobarbitone-anaesthetized and conscious rats (Wang & Pang, 1993a,b). MAP returned to the baseline level approximately 4 min after the injection of DPI and remained there during the 2 h observation period (Figure 7).

Baseline MAPs of rats before and 20 min after treatment with vehicle were 109 ± 2 and 112 ± 3 mmHg, respectively, which were similar to those of DPI-treated rats (10^{-5} mol kg^{-1}, i.v. bolus) (118 ± 5 and 114 ± 5 mmHg). Intravenous infusions of ACh and SNP caused dose-dependent depressor responses. Pretreatment with DPI significantly attenuated the depressor responses to ACh but not to SNP (Figure 8).

Discussion

Our *in vitro* results show that DPI selectivity inhibits endothelium-dependent relaxation induced by receptor-mediated (ACh) or non-receptor-mediated (A23187) mechanisms. The results are consistent with the report that DPI inhibits ACh-induced relaxation in the rabbit aorta (Stuehr *et al.*, 1991b) and further suggest there is no species difference for the actions of DPI. DPI also attenuates ACh- but not SNP-induced decreases in MAP in conscious rats, and ACh- but not SNP-induced vasodilatation in the perfused rat hindquarter preparation (unpublished observation). The results suggest that DPI inhibits endothelium-dependent vasodilatations in both conductance and resistance vessels. Therefore, the *in vitro* inhibitory effects of DPI on endothelium-dependent vasodilatations are similar to those of the N^G-substituted Arg analogues. These results are in accordance with the hypothesis that inhibition of NO synthesis causes the suppression of endothelium-dependent vasodilatation.

It has been known since 1973 that DPI suppresses the oxidation of NADH-like substrates thereby inhibiting mitochondrial oxidation (Holland *et al.*, 1973). It was later shown that DPI inhibits NADPH-dependent oxidase of neutrophils and macrophages (Cross & Jones, 1986; Hancock & Jones, 1987; Ellis *et al.*, 1988; 1989), and macrophage NO synthase (Stuehr *et al.*, 1991b), by specifically binding to and inhibiting the action of a plasma membrane polypeptide which may be a component of flavoprotein (Cross & Jones, 1986; Hancock & Jones, 1987; Ellis *et al.*, 1989). This suggests that flavin is the site of attack by DPI and that a protein is associated with FAD (O'Donnell *et al.*, 1993). Isoenzymes of NO synthase are known to be flavoproteins which contain FAD as a cofactor in the macrophage (Stuehr *et al.*, 1980; 1990; 1991a; Hevel *et al.*, 1991; White & Marletta, 1992), neutrophil (Yui *et al.*, 1991), brain (Mayer *et al.*, 1991; Lowenstein *et al.*, 1992; Bredt *et al.*, 1991; 1992; Hiki *et al.*, 1992) and liver (Evans *et al.*, 1992). There is, however, no functional documentation of a role for FAD as a cofactor of NO synthase in endothelial cells. Our *in vitro* results demonstrate that FAD interferes with both ACh-induced relaxation and the inhibitory effect of DPI on ACh-induced relaxation. The latter result, which is consistent with Stuehr *et al.*'s observation (1991b) that FAD antagonizes the inhibitory effect of DPI on macrophage NO synthesis, suggests that FAD and DPI may inhibit endothelial NO synthesis by a mechanism similar to that in macrophages. The former result is puzzling, since as a cofactor, FAD should facilitate rather than interfere with endothelium-dependent relaxation. FAD was indeed reported to facilitate macrophage NO synthesis (Stuehr *et al.*, 1990; Hevel *et al.*, 1991). The mechanism by which FAD inhibits ACh-induced relaxation is not clear at the moment, however, the effect may not be specific as FAD also inhibits SNP-induced relaxation (unpublished observation).

Our *in vitro* results also show that NADPH interferes with the inhibitory effect of DPI on ACh-induced relaxation. The antagonism of DPI by NADPH was specific since the same concentration of NADPH did not alter the inhibitory effect of L-NOARG. The inhibitory effect of DPI was also not affected by L-Arg, at a concentration previously found to reverse the inhibitory effects of L-NOARG and L-NAME on endothelium-dependent relaxations in aortic rings (Wang *et al.*, 1992; 1993). Our results with NADPH are consistent with those which show that both the constitutive (e.g. brain and endothelial) and inducible (e.g. macrophage and smooth muscle) NO synthases are dependent on NADPH as an essential cofactor (Mayer *et al.*, 1989; Stuehr *et al.*, 1989; 1990; 1991a; see McCall & Vallance, 1992). Regarding the nature of the interaction between NADPH and FAD, it has been suggested that NADPH suppresses the binding of DPI to the flavoprotein in neutrophil oxidase by preventing the attachment of DPI to a site in close proximity to the NADPH-binding site (Cross & Jones, 1986). It is very likely that NADPH may interfere with the action of DPI on endothelial NO synthase by the same mechanism.

Pretreatment with DPI was found to inhibit ACh-induced relaxation in aortic rings for at least 4 h after washout and to suppress ACh-induced vasodilatation for at least 2 h after intravenous bolus injection. Therefore, our *in vitro* and *in vivo* results are supportive of a prolonged inhibitory effect of DPI on endothelium-dependent vasodilatations. DPI has been reported to inhibit irreversibly macrophage NO synthase (Stuehr *et al.*, 1991b); the mechanism may involve the formation of a covalent bond with components of a flavoprotein (Ragan & Bloxham, 1977; O'Donnell *et al.*, 1993). However, our results show that post-treatment (1 h later) with NADPH still attenuates the effect of DPI, although the response is significantly less than that following pretreatment

(10 min earlier). These results may imply that fresh synthesis of NO occurs in endothelial cells.

It is well-known that all N^G-substituted Arg analogues which inhibit endothelium-dependent relaxation *in vitro* cause long-lasting pressor effects in whole animals. The pressor response of N^G-substituted Arg analogues is not blocked by the impairment of the central nervous system (Tabrizchi & Triggle, 1992; Wang & Pang, 1993a), sympathetic nervous system (Wang & Pang, 1991), renin-angiotensin system (Wang & Pang, 1991), or prostaglandin system (Rees *et al.*, 1989; Wang & Pang, unpublished data, 1993), but is inhibited by L-Arg (Aisaka *et al.*, 1989; Rees *et al.*, 1989; Wang & Pang, 1990; Wang *et al.*, 1991b; 1992). These observations have been accepted as evidence of a role of NO in the regulation of blood pressure (Rees *et al.*, 1989; Aisaka *et al.*, 1989; see Moncada *et al.*, 1991). As an 'irreversible' inhibitor of NO synthase, DPI should also cause a prolonged pressor response. However, unlike the N^G-substituted Arg analogues, intravenous bolus injections of DPI produced only immediate and transient increases in MAP. The pressor response of DPI was blocked by procedures which impair the activities of the central or sympathetic nervous systems, namely, pithing, spinal cord transection and the administration of tetrodotoxin, reserpine, guanethidine, phentolamine or prazosin (Wang & Pang, 1993a). Moreover, the pressor response to DPI, but not to L-NOARG, was accompanied by elevations of plasma noradrenaline and adrenaline (Wang & Pang, 1993a,b). These results show that the transient pressor response of DPI, unlike that of the N^G-substituted Arg analogues, is solely dependent on the activation of the sympathetic nervous system, i.e., DPI does not elicit a NO-dependent sustained rise in blood pressure as do the other NO synthase inhibitors.

Although one may postulate that the lack of effect of DPI is due to inadequate accumulation of drug *in situ* to inhibit NO synthesis, this is unlikely. DPI was shown to distribute rapidly and adequately to all organs or tissues (Gatley & Martin, 1979); moreover, its peak hypoglycaemic effect was reached at 1.5 h (Holland *et al.*, 1973) or 4 h (Gatley & Martin, 1979) after intraperitoneal injections, suggesting a long duration of action. Our present results also show that DPI inhibits irreversibly endothelium-dependent relaxation *in vitro* for more than 4 h, and partially inhibits ACh-induced vasodilatation *in vivo* even at 2 h after intravenous injection. It should be noted that a lack of complete inhibition of ACh-induced relaxation is a typical observation with NO synthase inhibitors since N^G-substituted Arg analogues, at maximal pressor doses, also cause partial inhibition of ACh-induced vasodilatation – this suggests that either the depressor/vasodilatation response of ACh *in vivo* is only partially due to the release of NO (Rees *et al.*, 1990; Wang *et al.*, 1992) or it is entirely independent of the biosynthesis and/or release of NO (Pang & Wang, 1993).

Although it is generally accepted that endogenous NO modulates vascular tone and blood pressure and that N^G-substituted Arg analogues produce pressor response by inhibition of endothelial NO synthesis and endothelium-dependent vasodilatations *in situ* (see Moncada *et al.*, 1991), our data with DPI suggest otherwise, i.e. inhibition of NO synthesis and endothelium-dependent vasodilatations do not always cause vasoconstriction *in vivo*. This hypothesis is supported by the recent publications which show that methylene blue does not produce a pressor response (Loeb & Longnecker, 1992; Pang & Wang, 1993) although it inhibits endothelium-dependent vasodilatation *in vitro* (Pang & Wang, 1993) and *in vivo* (Loeb & Longnecker, 1992). L-NOARG was also shown to cause much longer inhibition of endothelium-dependent vasodilatation than elevation of blood pressure in conscious rabbits, suggesting that the suppression of NO synthesis alone does not result in hypertension (Cocks *et al.*, 1992). Therefore, the hypothesis that endogenous NO modulates vascular tone and Arg analogues produce pressor response by inhibition of endothelial NO biosynthesis may need re-examination.

In summary, DPI efficaciously and 'irreversibly' inhibits endothelium-dependent vasodilatation *in vitro* and *in vivo* by a mechanism involving the suppression of the actions of FAD and NADPH. Unlike the N^G-substituted Arg analogues, DPI does not cause NO-mediated sustained pressor response. Instead, DPI causes immediate and transient pressor responses which are solely due to the activation of the sympathetic nervous system (Wang & Pang, 1993a,b). These results suggest that inhibition of NO synthesis *in situ* does not necessarily cause a pressor response.

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